Cell Stem Cell
Previews

Inhibition of Tgf-β Signaling Improves Mouse Fibroblast Reprogramming

Knut Woltjen[1,2,*] and William L. Stanford[1,3,4,*]
[1]Ontario Human Induced Pluripotent Stem Cell Facility, Toronto, ON M5G 1L7, Canada
[2]Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, ON M53 3H7, Canada
[3]Institute of Biomaterials and Biomedical Engineering, University of Toronto, Toronto, ON M5S 3G9, Canada
[4]Institute for Systems Biology, Seattle, WA 98103, USA
*Correspondence: woltjen@lunenfeld.ca (K.W.), william.stanford@utoronto.ca (W.L.S.)
DOI 10.1016/j.stem.2009.10.007

Circumventing genomic modification in induced pluripotent stem cell (iPSC) derivation is a clear requirement to realize safe clinical applications. In this issue of *Cell Stem Cell*, Ichida et al., (2009) describe a small molecule capable of replacing *Sox2*, offering insight into the reprogramming process.

Reactivation of the pluripotency network in somatic cells through transgenic overexpression of a defined set of transcription factors—*Oct4*, *Klf4*, *Sox2*, and *c-Myc*—leads to the generation of induced pluripotent stem cells (iPSCs) with embryonic stem cell (ESC)-like characteristics (Takahashi and Yamanaka, 2006). Although these cells hold immense promise in realizing personalized stem cell therapies, standard viral vectors result in permanent genomic modification, obstructing the safe application of iPSC derivatives. For therapeutic purposes, acceptable iPSC production techniques must make use of alternatives to oncogenes and/or employ transgenic methods free of permanent genomic modification (reviewed in O'Malley et al., 2009).

A major goal in the reprogramming field is to develop a cocktail consisting of chemical additives (and recombinant proteins, potentially) that can induce reprogramming of clinically accessible adult human cells at high efficiency, without the need for genetic factors (reviewed in Feng et al., 2009). To this end, a number of small molecules have been described that increase the efficiency of reprogramming. For example, histone deacetylase (HDAC) and methyltransferase (HMTase) inhibitors induce global changes in chromatin structure that are postulated to assist the reprogramming process by reducing activation thresholds to achieve endogenous gene expression patterns. In particular, valproic acid increases reprogramming efficiency to the point that rare iPSC lines can be established in the absence of *c-Myc* and *Klf4* (Huangfu et al., 2008). However, to replace *Oct4* and *Sox2*, genes occupying central nodes in the pluripotency transcriptional network, small molecules that activate the pluripotency network and directly or indirectly repress other transcriptional programs are required.

In this issue of *Cell Stem Cell*, Ichida et al. (2009) describe the results of a high-content chemical screen geared toward the discovery of molecules capable of reprogramming mouse fibroblasts in the absence of virally transduced *Sox2*. The lead molecule, E-616452 (RepSox, or Replacer of *Sox2*), a Transforming Growth Factor-β Receptor 1 (Tgfbr1/Alk5) kinase inhibitor, was capable of replacing the function of *Sox2* in MEFs infected with *Oct4*, *Klf4*, and *c-Myc* only, through a mechanism that did not involve direct activation of endogenous *Sox2* or *Sox2* family member expression. Antibody depletion of Tgf-β ligands and an anti-Tgf-βII antibody, as well as a nonspecific Tgfbr1 inhibitor (SB-431542; Alk-4, -5, and -7), resulted in similar levels of enhancement. iPSCs derived in the presence of RepSox were molecularly and functionally equivalent to iPSCs that arise from standard induction methods, contributing to adult chimeras, and the embryonic germline. Previously, it was observed that the small molecules BIX and BayK, HMTase and L-type calcium channel inhibitors, respectively, may also be used to replace *Sox2* in mouse fibroblast reprogramming (Shi et al., 2008). Thus, the isolation of specialized cell types already expressing endogenous reprogramming factors, such as *Sox2*-expressing neural progenitor cells, may not be necessary (Eminli et al., 2008).

In a similar report, Maherali and Hochedlinger (2009) demonstrated enhanced efficiency and kinetics of 4-factor reprogramming using the same Tgfbr1 inhibitors (E-616452/RepSox and SB-431542). This finding is contrary to Ichida et al. who observed only a minor impact on overall efficiency. While both groups utilized the same compounds, there were significant differences in their experimental designs, including the reprogramming induction strategies, compound concentrations, kinetics of compound application, and the assays quantifying reprogramming efficiency. Reassuringly, their critical findings are consistent despite these differences in the protocols used. Both groups report that inhibition of Tgf-β signaling early in reprogramming alleviated the need for transgenic *c-Myc* expression. Ichida et al. suggest this effect is not mediated through direct destabilization of the MEF transcriptional program but, rather, through activation of *L-Myc*, which can replace *c-Myc* in reprogramming of mouse fibroblasts (Nakagawa et al., 2008). Maherali and Hochedlinger also demonstrated that inhibition of Tgf-β signaling at early time points (days 1–3) during mouse fibroblast reprogramming could effectively substitute for transgenic *Sox2*. In contrast, Ichida et al. did not add Tgf-β inhibitors until at least day 4 of reprogramming and found that *Sox2* replacement by RepSox is a late event (beginning day 10 to day 11). Time-course analysis of RepSox treatment indicated that MEFs infected with *Oct4*, *Klf4*, and *c-Myc* required only a 24 hr pulse of RepSox on day 11 to finalize the reprogramming process, suggesting that RepSox acts by

triggering an endogenous switch in partially reprogrammed cells. Interestingly, neither group was able to simultaneously replace *c-Myc* and *Sox2* using inhibitors of Tgf-β signaling.

Using MEFs partially reprogrammed with only *Oct4*, *Klf4*, and *c-Myc*, Ichida et al. make a case for RepSox-mediated activation of *Nanog* through a series of correlative events. *Nanog* transcript levels increased following treatment with various Tgf-β inhibitors (RepSox, SB-431542, and anti-Tgf-β antibodies). However, endogenous Nanog expression is normally activated during reprogramming. Thus, is the high level of Nanog induction observed in partially reprogrammed MEFs 4 days after RepSox addition a by-product of stable reprogramming (i.e., the activation of the pluripotency transcriptional network) or a direct transcriptional activation of *Nanog* via inhibition of Tgf-β signaling as suggested by the authors? To support their hypothesis, the authors compared the ability of *Nanog* to substitute for *Sox2* in three-factor (*Oct4*, *Klf4*, and *c-Myc*) reprogramming experiments. While they found that *Nanog* and *Sox2* are interchangeable in primary inductions, this result is not surprising when one considers that OCT4, NANOG, and SOX2 function in a feed-forward autoregulatory motif to activate transcription of each other. Thus, this observation does not preclude the possibility that, rather than direct activation of *Nanog* by RepSox, the pathways mediated by NANOG and RepSox are parallel and converge elsewhere within the pluripotency network.

How might RepSox activate *Nanog* and induce the final stages of reprogramming in partially reprogrammed cells? Receptor-ligand interactions result in the activation or suppression of signaling networks that have broad, cell-context-dependent effects on gene expression. Ichida et al. (2009) noted that their partially reprogrammed cells exhibited high SMAD3 and activated *Id1*, *-2*, and *-3* upon exposure to RepSox, all hallmark Tgf-β signaling responses. They constructed a proposed signaling cascade built upon the Tgf-β-BMP/SMAD pathway that was gleaned from data generated from both human and mouse ESCs. While the critical nodes of the pluripotency transcriptional network are remarkably well conserved between mouse and humans, the signal inputs (growth factors and/or their small molecule replacers) into pluripotency networks are incongruent. Thus, it is not clear at this time whether the proposed signaling pathway is valid in the mouse reprogramming system; additional work will be required to fully elucidate the mechanism of RepSox action in reprogramming mouse fibroblasts to iPSCs. More importantly, what are the consequences of inhibition of Tgf-β signaling on human reprogramming? While Ichida et al. did not report any data from human experiments, Maherali and Hochedlinger (2009) commented that preliminary tests of Tgf-β inhibition had no effect on human reprogramming. If the mode of action of E-616452 is to activate the pluripotency network, then this finding would be predicted by the demonstration that BMP activation in the absence of Tgf-β/activin signaling promotes hESC differentiation (Xu et al., 2008). This point brings up a philosophical question of whether or not it is best to perform technically facile small molecule screens in the mouse and then analyze hits in humans, or directly perform the screens in humans? What percentage of the small molecules will maintain similar effects across species?

Extracting themes from these screens, on the other hand—such as those temporal events which impact cell state transitions, chromatin remodeling, suppression of differentiation, and maintenance of pluripotency—will direct the search for appropriate activators of equivalent pathways in human cells. The efforts of these and other groups have demonstrated that enhancer screens may be performed in the reprogramming scenario; perhaps focus should now be placed on translating these screening methodologies to a human platform. It may be that we never completely replace specific factors in the reprogramming process, as context is key in pathway activation and cell state transitions. Combinations of small molecules and protein transduction may eventually prove most promising.

REFERENCES

Eminli, S., Utikal, J., Arnold, K., Jaenisch, R., and Hochedlinger, K. (2008). Stem Cells *26*, 2467–2474.

Feng, B., Ng, J.H., Heng, J.C., and Ng, H.H. (2009). Cell Stem Cell *4*, 301–312.

Huangfu, D., Osafune, K., Maehr, R., Guo, W., Eijkelenboom, A., Chen, S., Muhlestein, W., and Melton, D.A. (2008). Nat. Biotechnol. *26*, 1269–1275.

Ichida, J.K., Blanchard, J., Lam, K., Son, E.Y., Chung, J.E., Egli, D., Loh, K.M., Carter, A.C., Di Giorgio, F.P., Koszka, K., et al. (2009). Cell Stem Cell *5*, this issue, 491–503.

Maherali, N., and Hochedlinger, K. (2009). Curr. Biol., in press. Published online September 16, 2009. 10.1016/j.cub.2009.08.025.

Nakagawa, M., Koyanagi, M., Tanabe, K., Takahashi, K., Ichisaka, T., Aoi, T., Okita, K., Mochiduki, Y., Takizawa, N., and Yamanaka, S. (2008). Nat. Biotechnol. *26*, 101–106.

O'Malley, J., Woltjen, K., and Kaji, K. (2009). Curr. Opin. Biotechnol., in press. Published online October 16, 2009. 10.1016/j.copbio.2009.09.005.

Shi, Y., Desponts, C., Do, J.T., Hahm, H.S., Schöler, H.R., and Ding, S. (2008). Cell Stem Cell *3*, 568–574.

Takahashi, K., and Yamanaka, S. (2006). Cell *126*, 663–676.

Xu, R.-H., Sampsell-Barron, T.L., Gu, F., Root, S., Peck, R.M., Pan, G., Yu, J., Antosiewicz-Bourget, J., Tian, S., Stewart, R., and Thomson, J.A. (2008). Cell Stem Cell *3*, 196–206.